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(54) Title: CULTURED INNER CELL MASS CELL LINES DERIVED FROM UNGULATE EMBRYOS

(57) Abstract

Novel cultured inner cell mass (CICM) cells, and cell lines, derived from ungulates, in particular, pigs and cows, and methods for their preparation are provided. The subject CICMs possess similar morphology and express cell markers identically or substantially similarly to ICMs of undifferentiated developing embryos for prolonged culturing periods. Heterologous DNAs may be introduced into the subject CICM cells and cell lines to produce transgenic cells useful for the production of transgenic embryos, fetuses and/or offspring, e.g., by nuclear transfer procedures.

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CULTURED INNER CELL MASS CELL LINES
DERIVED FROM UNGULATE EMBRYOS

5   Field of the Invention
        Novel cultured inner cell mass (CICM) cells, cell
lines, and methods for their preparation are provided. The
subject CICMs possess similar morphology and express cell
markers identically or highly similarly to ICMs of develop-
10   ing embryos for prolonged culturing periods. These CICMs
are produced by novel culturing techniques and/or by intro-
duction of a regulatable differentiation inhibiting gene
(DI). The subject CICM cell lines are used to produce
differentiated cells, tissues, organs and/or whole animals,
15   preferably ungulates, desirably those which have been genet-
ically modified to contain within their genome a desired
heterologous DNA or which have been selected to contain
genetically desirable traits. This is accomplished by
in vitro culture techniques or by producing chimeric or
20   nuclear transfer embryos, fetuses and/or offspring. More-
over, the CICM cells can also be used for cloning (nuclear
transfer procedures) to produce genetically identical
embryos, fetuses and/or offspring.

25   Background of the Invention
        Methods for deriving embryonic stem (ES) cell lines
in vitro from early preimplantation mouse embryos are well
known. (*See*, e.g., Evans et al., Nature, 29:154-156 (1981);
Martin, Proc. Natl. Acad. Sci., USA, 78:7634-7638 (1981)).
30   ES cells can be passaged in an undifferentiated state,
provided that a feeder layer of fibroblast cells (Evans
et al., Id.) or a differentiation inhibiting source (Smith
et al., Dev. Biol., 121:1-9 (1987)) is present.
        ES cells have been previously reported to possess
35   numerous applications. For example, it has been reported
that ES cells can be used as an *in vitro* model for differen-
tiation, especially for the study of genes which are

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involved in the regulation of early development. Mouse ES
cells can give rise to germline chimeras when introduced
into preimplantation mouse embryos, thus demonstrating their
pluripotency (Bradley et al., Nature, 309:255-256 (1984)).

5   In view of their ability to transfer their genome to
the next generation, ES cells have potential utility for
germline manipulation of livestock animals by using ES cells
with or without a desired genetic modification. Moreover,
in the case of livestock animals, e.g., ungulates, nuclei

10   from like preimplantation livestock embryos support the
development of enucleated oocytes to term (Smith et al.,
Biol. Reprod., 40:1027-1035 (1989); and Keefer et al., Biol.
Reprod., 50:935-939 (1994)). This is in contrast to nuclei
from mouse embryos which beyond the eight-cell stage after

15   transfer reportedly do not support the development of
enucleated oocytes (Cheong et al, Biol. Reprod 48:958
(1993)). Therefore, ES cells from livestock animals are
highly desirable because they may provide a potential source
of totipotent donor nuclei, genetically manipulated or

20   otherwise, for nuclear transfer procedures.
        Many research groups have reported the isolation of
purportedly pluripotent embryonic cell lines. For example,
Notarianni et al., J. Reprod. Fert. Suppl., 43:255-260
(1991), report the establishment of purportedly stable,

25   pluripotent cell lines from pig and sheep blastocysts which
exhibit some morphological and growth characteristics simi-
lar to that of cells in primary cultures of inner cell
masses isolated immunosurgically from sheep blastocysts.
(Id.) Also, Notarianni et al., J. Reprod. Fert. Suppl.,

30   41:51-56 (1990) discloses maintenance and differentiation in
culture of putative pluripotential embryonic cell lines from
pig blastocysts. Further, Gerfen et al., Anim. Biotech,
6(1):1-14 (1995) disclose the isolation of embryonic cell
lines from porcine blastocysts. These cells are stably

35   maintained in mouse embryonic fibroblast feeder layers
without the use of conditioned medium. These cells report-

edly differentiate into several different cell types during
culture (Gerfen et al., Id.).

Further, Saito et al., Roux's Arch. Dev. Biol.,
201:134-141 (1992) report bovine embryonic stem cell-like
5   cell lines cultured which survived passages for three, but
were lost after the fourth passage. Still further, Handy-
side et al., Roux's Arch. Dev. Biol., 196:185-190 (1987)
disclose culturing of immunosurgically isolated inner cell
masses of sheep embryos under conditions which allow for the
10 isolation of mouse ES cell lines derived from mouse ICMs.
Handyside et al. (1987) (Id.), report that under such condi-
tions, the sheep ICMs attach, spread, and develop areas of
both ES cell-like and endoderm-like cells, but that after
prolonged culture only endoderm-like cells are evident.
15 (Id.)

Recently, Cherny et al., Theriogenology, 41:175 (1994)
reported purportedly pluripotent bovine primordial germ
cell-derived cell lines maintained in long-term culture.
These cells, after approximately seven days in culture,
20 produce ES-like colonies which stain positive for alkaline
phosphatase (AP), exhibit the ability to form embryoid
bodies, and spontaneously differentiate into at least two
different cell types. These cells also reportedly express
mRNA for the transcription factors OCT4, OCT6 and HES1, a
25 pattern of homeobox genes which is believed to be expressed
by ES cells exclusively.

Also recently, Campbell et al., Theriogenology, 43:181
(1995) in an abstract report the production of live lambs
following nuclear transfer of cultured embryonic disc (ED)
30 cells from day nine ovine embryos cultured under conditions
which promote the isolation of ES cell lines in the mouse.
The authors conclude based on their results that ED cells
from day nine ovine embryos are totipotent by nuclear trans-
fer and that totipotency is maintained in culture for up to
35 three passages.

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Even more recently, Campbell et al, Nature, 380:64-68
(1996) reported cloning of sheep by nucleic transfer from a
cultured cell line. The cells used are unlike the CICM's of
the present invention. Unlike the subject CICM cells, the
5   cells of Campbell et al formed a monolayer in tissue cul-
ture. The authors refer to these cells as being "flattened"
or as exhibiting an "epithelial" appearance. By contrast,
the CICM cells of the present invention can be continually
maintained in a multilayer colony or portions of the colony
10   when grown in an undifferentiated state. Also, the cells of
Campbell et al are cytokeratin and are laminin A/C positive.
By contrast, the CICM cells of the present invention are
cytokeratin negative.

Moreover, there is no suggestion that the cells of
15   Campbell et al are undifferentiated. Rather, the reference
only indicates that these cells are useful in nucleic trans-
fer procedures. Also, these cells are not cultured under
conditions wherein they maintain constant contact with a
fibroblast feeder layer. Rather, the cultured cells (of
20   Campbell et al (1996) apparently push the fibroblasts to the
side in culture and grow on top of the culture dish.

Van Stekelenburg-Hamers et al., Mol. Reprod. Dev.,
40:444-454 (1995), reported the isolation and characteriza-
tion of purportedly permanent cell lines from inner cell
25   mass cells of bovine blastocysts. The authors isolated and
cultured ICMs from 8 or 9 day bovine blastocysts under
different conditions to determine which feeder cells and
culture media are most efficient in supporting the attach-
ment and outgrowth of bovine ICM cells. They concluded
30   based on their results that the attachment and outgrowth of
cultured ICM cells is enhanced by the use of STO (mouse
fibroblast) feeder cells (instead of bovine uterus epitheli-
al cells) and by the use of charcoal-stripped serum (rather
than normal serum) to supplement the culture medium. Van
35   Stekelenburg et al report, however, that their cell lines

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resembled epithelial cells more than pluripotent ICM cells.
(Id.)

        Still further, Smith et al., WO 94/24274, published
October 27, 1994, Evans et al, WO 90/03432, published April
5, 1990 and Wheeler et al, WO 94/26889 published November
24, 1994 report the isolation, selection and propagation of
animal stem cells which purportedly may be used to obtain
transgenic animals. Also, Evans et al., WO 90/03432, pub-
lished on April 5, 1990, report the derivation of purported-
ly pluripotent embryonic stem cells derived from porcine and
bovine species which assertedly are useful for the produc-
tion of transgenic animals. Further, Wheeler et al.,
WO 94/26884, published November 24, 1994, disclose embryonic
stem cells which are assertedly useful for the manufacture
of chimeric and transgenic ungulates. Thus, based on the
foregoing, it is evident that many groups have attempted to
produce ES cell lines, e.g., because of their potential
application in the production of cloned or transgenic
embryos and in nuclear transplantation.

        The use of ungulate ICM cells for nuclear transplanta-
tion has also been reported. For example, Collas et al.,
Mol. Reprod. Dev., 38:264-267 (1994) disclose nuclear trans-
plantation of bovine ICMs by microinjection of the lysed
donor cells into enucleated mature oocytes. The reference
discloses culturing of embryos in vitro for seven days to
produce fifteen blastocysts which, upon transferral into
bovine recipients, resulted in four pregnancies and two
births. Also, Keefer et al., Biol. Reprod., 50:935-939
(1994), disclose the use of bovine ICM cells as donor nuclei
in nuclear transfer procedures, to produce blastocysts
which, upon transplantation into bovine recipients, resulted
in several live offspring. Further, Sims et al., Proc.
Natl. Acad. Sci., USA, 90:6143-6147 (1993), disclose the
production of calves by transfer of nuclei from short-term
in vitro cultured bovine ICM cells into enucleated mature
oocytes.

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Also, the production of live lambs following nuclear
transfer of short-term cultured embryonic disc cells (up to
three passages) has been reported (Campbell et al., <u>Therio-
genology</u>, 43:181 (1995)). Still further, the use of bovine
5   pluripotent embryonic cells in nuclear transfer and the
production of chimeric fetuses has also been reported (Stice
et al., <u>Theriogenology</u>, 41:301 (1994)).

However, notwithstanding what has been previously
reported in the literature, there still exists a significant
10   need for cultured ICM cells and cell lines which possess
improved properties, e.g., which possess morphological
properties and express cell markers identically or substan-
tially similar to ICM cells of developing embryos, in par-
ticular ungulate embryos. There further exists a signifi-
15   cant need in the art for methods of producing such improved
cultured ICM cells and cell lines.

Objects of the Invention

It is therefore an object of the invention to provide
20   novel and improved cultured inner cell mass (ICM) cells and
cell lines.

It is a more specific object of the invention to pro-
vide novel and improved cultured ICM cells and cell lines
which exhibit morphological characteristics and express cell
25   markers identically or substantially similarly to that of
the ICM of developing embryos.

It is an even more specific object of the invention to
provide novel and improved ungulate cultured ICM cells and
cell lines which exhibit morphological characteristics and
30   express cell markers identically or substantially similarly
to that of ICM of developing ungulate embryos.

It is another specific object of the invention to
provide improved cultured ICM cells and cell lines, prefer-
ably derived from ungulates, which exhibit morphological
35   characteristics and which express cell markers identically

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or substantially similarly to ICM of developing ungulate
embryos, for prolonged culturing periods.

It is another specific object of the invention to
provide novel methods for the isolation and/or production of
5   such improved ungulate cultured ICM cells and cell lines.

It is a more specific object of the invention to pro-
vide novel methods for the isolation and/or production of
cultured ungulate ICM cells or cell lines which exhibit
morphological characteristics and which express cell markers
10   identically or substantially similarly to the ICM of devel-
oping ungulate embryos, preferably for long periods in
culture.

It is a specific object of the invention to provide a
novel method for culturing and selecting ICM cells or cell
15   lines which exhibit morphological characteristics and
express cell markers identically or substantially similarly
to ICMs of developing ungulate embryos, which method com-
prises:

(i)   obtaining an ICM of a blastocyst or ICM progeni-
20   tor cells from preblastocyst stage embryos by mechanical
and/or enzymatic means;

(ii) culturing said ICM on a feeder layer culture,
preferably fibroblasts; and

(iii) identifying from among said cultured cells those
25   cells which exhibit the following properties;

(a)   small cytoplasmic/nuclear volume ratio;

(b)   cytoplasmic vesicles; and

(c)   small individual cells;

(iv) separating cells having such properties from the
30   remaining cultured cells; and

(v)   passaging said separated cells onto a feeder
layer, preferably fibroblasts, under conditions such that
the separated cells are in direct physical contact with the
feeder layer.

35   It is a more specific object of the invention to pro-
duce improved cultured ICMs by the following steps:

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(i)   obtaining an ICM of blastocyst stage embryos,
preferably ungulates, by suitable mechanical and/or enzyma-
tic means;

(ii)   culturing said obtained ICM onto a confluent,
5   preferably thick, feeder cell monolayer preferably comprised
of fibroblast cells;

(iii)   culturing said ICM under conditions which result
in a multilayer cell colony which includes a substantially
inner population of flattened epithelial-like cells and a
10   second multilayered population of cells which substantially
surrounds said inner epithelial-like cells and includes
relatively small cells having cytoplasmic vesicles, and
small cytoplasmic/nuclear volume ratios;

(iv)   separating said second multilayered population of
15   cells which are substantially comprised on the perimeter of
the multilayer cell colony by suitable non-degradative
means, i.e., mechanical and/or enzymatic means; and

(v)   passaging said separated cells onto a new feeder
layer preferably comprised of fibroblasts under conditions
20   such that the separated cells are in direct physical contact
with the feeder layer.

It is another object of the invention to provide cul-
tured ICMs which exhibit morphological characteristics and
which express cell markers identically or substantially
25   similar to that of ICMs of developing ungulate embryos by a
method comprising:

(i)   obtaining ungulate ICM cells or an established
cultured ungulate ICM cell line;

(ii)   introducing into the nucleus of said ICM cells or
30   said established cultured ICM cell line one or more genes
which inhibit differentiation, (DI genes) which DI gene or
DI genes are preferably expressed under the control of an
inducible promoter;

(iii)   culturing the resultant transgenic ICM cells or
35   cultured ICM cell line on a feeder layer, preferably a
confluent fibroblast layer, under conditions which provide

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for the expression of said differentiation inhibiting gene or genes.

It is also an object of the invention to use ICM cells or cell lines which express one or more DI genes in the above-described culturing method.

It is another specific object of the invention to use the subject improved cultured ICMs which exhibit morphological characteristics and express cell markers identically or substantially similarly to ICMs of developing ungulate embryos for any usage wherein ICMs or cultured ICMs have applicability. Such usages include, e.g., the production of differentiated cells, tissues, organs and/or whole animals by *in vitro* cell culture techniques or the production of chimeric or nuclear transfer embryos which may or may not be transgenic.

It is a specific object of the invention to provide cultured ICM cells which may be used in cloning (nuclear transfer procedures) to produce genetically identical ungulate embryos, fetuses and/or offspring or to produce chimeric ungulate embryos, fetuses or offspring.

It is another specific object of the invention to use the subject cultured ICMs or cell lines derived therefrom for integration of desired heterologous DNAs, and to use the resultant transgenic cultured ICMs to produce transgenic ungulate embryos, fetuses, and/or offspring or to produce transgenic chimeric ungulate embryos, and/or offspring.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a photograph of cultured CICM cells grown without feeder layer contact. Embryoid bodies may be observed.

Figure 2 is a photograph of cytokeratin positive cultured CICM cells.

Figure 3 is a photograph of CICM cells on a fibroblast feeder layer. Multiple layer colonies are visible after only 2 days of culturing.

Figures 4 and 5 are photographs showing AP positive and cytokeratin negative CICM cell colonies.

Figures 6 and 7 are photographs showing epithelial-like cells which are obtained during culturing of CICM cells.

5   Those cells are AP negative and cytokeratin positive.

Figure 8 is a photograph of CICM cell colonies. This photo shows that multilayer colonies are beginning to flatten into epithelial-like cell sheets. The cells in the middle of the colony are AP negative and exhibit a flattened

10   epithelial-like appearance. By contrast, cells in the perimeter are smaller, exhibit a multilayered morphology and possess cytoplasmic vesicles.

Figure 9 is a photograph showing cultured pig CICM cells expressing a beta-galactosidase DNA construct five

15   days after microinjection.

DETAILED DESCRIPTION OF THE INVENTION

Prior to discussing the invention in more detail, the following definitions are provided.

20   **Inner cell mass cells (ICM cells)**: This is one of two distinct cell types which are produced during early embryonic development, i.e., the blastocyst and eventually forms part of the feeder. These cells have known application in nuclear transfer techniques, and for producing chimeric and

25   cloned offspring.

Trophectoderm (TE cells): This refers to the second of two distinct cell types which are produced during early embryonic development, i.e., the blastocyst stage and eventually forms part of the placenta.

30   **ICM progenitor cells**: These are cells comprised in pre-blastocyst stage embryos which develop into ICM cells.

Cultured inner cell mass cells: This refers to inner cell mass cells which have been cultured in vitro for a prolonged period of time.

35   **Cultured inner cell mass (CICM or cultured ICM) are cells which exhibit morphological characteristics and which**

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express cell markers identically or substantially similarly to inner cell mass cells of developing embryos: In the present invention, this will refer to cultured ICM cells which exhibit a morphology identical or highly similar to
5 the ICM of developing embryos, e.g., ungulate embryos. In general, such cells will grow as small multilayer colonies; however, some cells may differentiate if the colony size exceeds approximately 50 to 100 cells.

CICMs which express cell markers identically to ICMs of
10 developing ungulate embryos refers to CICM cells which express or do not express cell markers in a manner which is characteristic of undifferentiated ICMs of developing ungu-late embryos. Suitable cell markers which may be used to identify suitable CICMs include by way of example cytokera-
15 tins, in particular cytokeratin 8 and cytokeratin 18, enzymes such as alkaline phosphatase, and other cell markers expressed in ICMs such as rex-1, 1 amin ac, and oct4. Ideally, the levels of expression (if any) of such cell markers will be the same as in undifferentiated ICMs ob-
20 tained from ungulate embryos.

CICMs which express cell markers substantially similar-ly to ICMs of developing ungulate embryos refers to CICMs which express a majority of cell markers which are charac-teristic of undifferentiated developing ungulate ICM
25 embryos, e.g., cytokeratins such as cytokeratin 18 and enzymes such as alkaline phosphatase, and other cell markers such as rex-1 ac and oct4. Substantially similar refers to the fact that the amount of expression of some cell markers may vary, and some cell markers may be expressed differently
30 in the subject CICMs than in undifferentiated ICMs of ungu-late embryos provided that this does not adversely affect the ability of the resultant CICMs to be cultured and main-tained according to the invention.

In general, CICM cells which express cell markers
35 identically or substantially similarly to ICMs of developing ungulate embryos will not express cytokeratin 18, and will

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express alkaline phosphatase. Methods for detecting the
expression of such cell markers and others are known in the
art (some of which are referenced *infra*) and include by way
of example immunodetection methods.   For example, such

5   methods detect the expression or lack of expression of a
particular cellular marker based on reactivity of cells with
a suitable immunoprobe, e.g., a labeled antibody which pro-
vides for specific detection.

However, as discussed *infra*, there may be species

10   differences in cell marker expression.   (For example, where-
as CICMs obtained from pigs are AP positive, CICMs obtained
from cows are predominantly AP negative.) Moreover, the
cultured ICMs of the present invention may also contain
genes which are not normally contained in ICMs, e.g., genes

15   which encode for a desired product and/or one or more genes
which inhibit differentiation.

Differentiation inhibiting gene: In the present inven-
tion this will typically refer to any nucleic acid sequence
which inhibits the differentiation of ICMs.   This includes

20   by way of example tsA58 as well as other genes encoding
other T antigens and oncogene products, cytokines and tran-
scription factors, e.g., OCT3, LIF and LIF receptor.   Such
differentiation inhibiting genes are known in the art and
are described in WO 91/13150; Okamoto et al., Cell, 60:461

25   (1990); Rosner et al., Nature, 345:686 (1990); and Smith
et al., Nature, 336:688 (1988), all of which are incorporat-
ed by reference in their entirety herein.   Other suitable
genes include REX-1 (Rodgers et al, Develop. 113:815-824
(1991)), and FGF-5 (Herbert et al, Develop. 112:407-415

30   (1991).)

Inducible or regulatable promoter: This refers to any
promoter which, when operably linked to a desired structural
gene, e.g., a differentiation inhibiting gene, is "turned
on", i.e., promotes transcription, under specific condi-

35   tions. Typically, this requires the presence or absence of
one or more substituents in the culture medium, e.g., metal

ions, or other specific culturing conditions, e.g., particu-
lar temperature conditions, etc. Examples of well known
inducible or regulatable promoters include by way of example
response elements such as tetracycline (WO 94/29442), inter-
5   feron (Kimura et al., _Cell_, 44:261 (1986)), steroid and
metallothionein promoters (Yarranton, G.T., _Curr. Opin._
Biotech., 3:506 (1992)), temperature inducible promoters,
etc. These references are incorporated by reference in
their entirety.

10   **Feeder cells**: This refers to any cells which can be
used to obtain and/or propagate undifferentiated cultured
ICM cell lines. Preferably, such feeder cells will be
fibroblasts, and more preferably murine embryonic fibro-
blasts, e.g., from 12-16 day old murine fetuses. Other
15 suitable feeder cells include, e.g., fibroblast and uterine
epithelial cells from ungulates, chicken fibroblasts, rat
fibroblast, STO and SI-m220 feeder cell lines, and BRL
cells.

     Cultured multilayer ICM colony: This refers to a
20 growing multilayer colony of cultured ICMs on the feeder
layer which exhibits a multilayer structure possessing two
different, distinct cell populations. The first cell popu-
lation substantially constitutes the perimeter of the multi-
layer cell colony and is multilayered. The cells therein
25 include cells which are relatively small, possess cytoplas-
mic vesicles, and which stain strongly positive for AP
activity. The other cell population is substantially com-
prised in the middle of the cell colony and substantially
consists of a flattened epithelial-like population of cells
30 which exhibit little or no AP activity.

     As discussed, the present invention is generally di-
rected to cultured ICM cells and cell lines which exhibit
improved properties in relation to previously reported
cultured ICMs. In particular, these cultured ICMs and cells
35 exhibit morphological characteristics and express cell

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markers identically or substantially similarly to the ICMs
of developing embryos.

The present invention provides cultured ICMs which
possess a novel combination of properties which are identi-
5   cal or substantially similar to ICMs of developing ungulate
embryos, i.e., they possess the above-defined multilayer
cell colony morphology, and express cell markers identically
or substantially similarly to ICMs of developing ungulate
embryos, e.g., they do not express cytokeratin 18 and may or
10   may not express alkaline phosphatase (depending on particu-
lar species of origin.)

Both the alkaline phosphatase marker and cytokeratin 18
marker have been used independently by previous researchers
to determine whether cultured cells are putatively similar
15   to developing ungulate ICMs (Piedrahita et al., Theriogenol-
ogy, 34:879 (1990), Wheeler et al., Reprod. Fert. Dev.,
6:563 (1994) and Talbot et al., Mol. Reprod. Dev., 36:139
(1993)). Thus, the expression of these cell markers by the
CICMs produced herein provides strong evidence that the
20   subject culturing techniques may be used to obtain ICMs
which are identical or substantially similar to ICMs found
in developing ungulate embryos.

This is in contrast to previously disclosed cultured
ICMs, such as the cultured pig ICMs of Talbot et al (Id.)
25   which, after culturing for only two weeks in vitro, differ-
entiated and lost AP activity. The subject cultured ICMs
are also different from the cultured bovine ICM cells of
Sims et al. (Id.), which were cultured for short periods of
time but which were disaggregated and grown as individual
30   cells in a cell suspension system. The subject cultured
ICMs are further different from ES-like cells which have
. been previously described in the literature which grow as an
epithelial monolayer and which are AP negative and cytokera-
tin positive. Further, the subject cultured ICMs are dif-
35   ferent from those of Wheeler, Reprod. Fert. Dev., 6:563
(1994) and WO 94/26884 which, while growing in multilayer

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colonies and being cytokeratin negative (similar to CICM cell lines of the present invention) are feeder layer independent.

Thus, the subject invention provides novel CICM cells and cell lines which, given their morphological and cell marker characteristics, should be well suited for chimera and nuclear transfer studies to produce differentiated cells, fetuses and offspring. In general, the subject novel CICM cell lines are produced by either of the following two methods.

The first method comprises obtaining ICMs of blastocyst or ICM progenitor cells from preblastocyst stage embryos, preferably those derived from ungulates. Ungulates include many important livestock animals, e.g., swine, cattle, sheep, horses and goats. Therefore, cultured ungulate cells potentially suitable for the production of ungulate animals are highly desirable. Also, ungulates afford significant advantages over other species known to be useful for production of cultured ICMs and transgenic or cloned animals, e.g., rodents, because they are immunologically and physiologically more similar to humans.

Ungulate embryos of blastocyst or preblastocyst stage may be obtained by well known methods. For example, blastocyst or preblastocyst embryos may be surgically collected from the reproductive tract of ungulates, e.g., pigs or cows, post mortem during surgical laparotomy, or non-surgically. Generally these embryos will range in age from 2 to 15 days and preferably 8 days. After collection, the ICMs of the blastocyst or preblastocyst stage ungulate embryos will either be partially separated (e.g. ICM separated from the trophoblast cells) or left intact. If partial separation is effected, it is typically effected by suitable mechanical and/or enzymatic methods, e.g., by use of a culturing glass needle and/or by incubation with trypsin or pronase.

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The partially separated or intact ICMs of the blasto-
cyst which contains the ICM and at least a portion of the
trophectoderm or ICM progenitor cells derived from pre-
blastocyst stage embryos are then introduced onto a suitable
5   feeder cell layer culture medium. All cells derived from
preblastocyst stage embryos are introduced onto a suitable
feeder layer. As discussed *supra*, the feeder cell layer
will comprise any cell layer which allows for the selection
and/or propagation of undifferentiated ICMs. Preferably,
10   the feeder cell layer will comprise fibroblasts, and more
preferably those derived from primary cultures of embryonic
murine fibroblasts. However, it is expected that fibroblast
cell lines or other types of fibroblasts may be substituted
therefor.

15   It has been found by the present inventors that the
morphological characteristics of the feeder layer is an
important factor in obtaining and propagating undifferenti-
ated CICM cell lines in culture. More particularly, it has
been found that the culture plate used for culturing the
20   ICMs should preferably comprise a thick confluent monolayer
of feeder cells, more preferably a thick confluent monolayer
of murine fibroblast cells.

As discussed in greater detail in the Examples, the
feeder layer is preferably obtained from primary cultures of
25   embryonic fibroblasts, e.g., cells derived from 12-16 day
old murine fetuses. Methods for the isolation of fibro-
blasts are well known in the art. For example, fibroblasts
may be collected by aseptic removal of the head, liver,
heart and alimentary tract from suitable murine fetuses,
30   which are then minced and incubated under suitable condi-
tions to provide for cell dissociation, e.g., incubation
with a trypsin containing composition, and the dissociated
fibroblasts then plated onto tissue culture dishes contain-
ing suitable culture medium.

35   Any medium suitable for maintaining the cultured feeder
cells, e.g., murine fibroblasts, may be used.   In partic-

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ular, the present inventors chose to plate fibroblast cells on tissue culture dishes and culture in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logen, UT), penicillin (100

5   IU/ml) and streptomycin (50 μg/ml). However, it is expected that other media may be substituted therefore, including, e.g., DMEM supplemented with glutamine, glucose, 2-mercapto, ethanol, MEM non-essential amino acids, 5-20% serum, anti-bodies, nucleosides, glutamine (See Strojek et al, Therio-

10   genology 33:981 (1990); Notarianni et al., J. Reprod. Fert. 43 (Suppl): 255 (1990)); and CM beta and BRL conditioned medium (See Handyside et al, Roux's Arch. Dev. Biol., 196:185 (1987)).

After the fibroblasts are cultured to confluence, they

15   are then passaged onto other tissue culture dishes or used directly for culturing of ICM cells and cell lines. Prefer-ably, at some time after passage, and prior to introduction of a ICM, the feeder cells, e.g., fibroblasts, are also treated with an amount of an antibiotic, e.g., mytomycin C,

20   preferably from 5 to 100 μg/ml, and more preferably about 10 μg/ml of mytomycin C, contained in a suitable culture medium, e.g., alpha-MEM or exposed to irradiation to stop or impede the growth of fibroblasts.

The feeder cells, e.g., fibroblasts are preferably

25   cultured under conditions which allow for the production of a thick confluent monolayer of cells on the culture dishes. For example, this may be effected by maintaining the fibro-blasts in a humidified atmosphere, e.g., one containing 5% CO_2 in air at 37°C. However, it is expected that the spe-

30   cific culturing conditions may be varied dependent upon factors including, e.g., the type of feeder cells, age, species, among other factors.

As discussed, culture plates which contain the desired feeder layer, preferably in the form of a thick confluent

35   cell monolayer, are then used to obtain and culture the subject CICM cells and cell lines. This will preferably

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comprise plating the partially separated or intact ungulate
ICMs containing at least a portion of the trophectoderm
directly onto the mytomycin C treated confluent feeder
layer. This is effected by any means which provides for

5   direct physical contact between the cultured ICM and the
feeder cells. This may be accomplished by different meth-
ods. For example, this may be effected by use of a glass
pipette to initiate contact between the ICM and the fibro-
blast feeder layer. Alternatively, physical contact between

10 feeder cell layer and cultured ICMs may be effected by
placing the ICM cells under the feeder layer and the bottom
of the culture dish, or by centrifuging ICM cells so as to
force them onto the feeder cell layer.

The ICMs are cultured on the feeder layer using any

15 culture medium which allows for the growth and maintenance
of ICMs and the desired multilayer colony morphology.
Preferably, the CICM cells or cell lines will be maintained
in a growth medium consisting of alpha-MEM supplemented with
FCS and 0.1 mM beta-mercaptoethanol (Sigma). However,

20 other culture medium may be substituted therefore including,
e.g., the cultured media disclosed supra.

The growth medium is exchanged for fresh medium as
necessary during culturing so as to optimize cell growth.
This is typically effected about every 2-3 days. However,

25 this may vary dependent upon the specific feeder cells and
the selected culture medium. After culturing is effected
for several days, usually about 4 days, the first cultured
ICM or CICM colonies will be observed, and some time there-
after, typically at least about 1 day later, the cultured

30 ICMs may then be passaged onto other fibroblast feeder layer
containing culture plates.

It has also been found that the passaging efficiency of
CICM cells is enhanced when they are passaged together with
some associated feeder cells onto a new feeder layer.

35 Therefore, the new passage contains some of the feeder cells
from the previous passage. In passaging the CICM cells

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together with some associated feeder cells (fibroblasts), it has been found that the passaging efficiency (percentage of CICM cell clumps which result in new colonies) is significantly enhanced.

5   As discussed above, an important part of the invention comprises the discovery that CICM cells comprising a particular combination of morphological properties are preferred for passaging and the production of cultured ICM cells and cell lines having the desired properties. Specifically,

10   cells which possess the following morphological characteristics are preferred:

(i)   small cytoplasmic/nuclear volume ratio (ranging from about 10/90 to about 50/50, more preferably about 10/90 to about 30/70, and most preferably about 25/75);

15   (ii) observable cytoplasmic vesicles; and

(iii) small individual cells, ranging from about 10 - 20 μm in diameter, and preferably less than about 15 μm in diameter.

Calculation of cytoplasmic/nuclear volume ratios may be

20   easily determined by observing cultured cells under the microscope, taking appropriate measurements, and making appropriate volumetric calculations. Similarly, cytoplasmic vesicles can easily be observed in cultured cells. Finally, cell size can be easily determined by measuring cell diame-

25   ters of CICMs comprised on the feeder layer culture.

It has been surprisingly discovered by the present inventors that these morphological properties are important for the isolation and propagation of cultured ICM cells and the production of cell lines having the desired morphologi-

30   cal and cell marker characteristics and which maintain such properties for prolonged periods in tissue culture, i.e., .after repeated passaging.

More specifically, the present invention was based on the observation that when ICM or passaged CICM cell lines

35   initially attach to the feeder layer, multiple layer colonies shortly become visible, typically after about 2 days.

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However, these multilayer colonies generally begin to flat-
ten into epithelial sheets of cells as the cells propagate
in vitro. Related to this observation, it was discovered
that while the cells contained in the multilayer section of
5   the colony are AP positive and cytokeratin 18 negative, the
flattened epithelial-like cells are AP negative and cyto-
keratin 18 positive. Thus, the epithelial-like cells ex-
press cell markers differently from ICMs of developing
fetuses. Accordingly, it was discovered that ICMs cultured
10   on feeder cell layers over time gradually exhibit a morphol-
ogy and express cell markers which are inconsistent with
ICMs of undifferentiated developing embryos. This is unde-
sirable because CICMs which exhibit properties identically
or substantially similarly to undifferentiated ICMs of
15   developing embryos will potentially be totipotent and there-
fore should be useful in chimeric and nuclear transfer (NT)
techniques.

      Thus, the goal of the present invention was to develop
culturing methods which maintain or revert these ICM cell
20   colonies such that they comprise the desired multilayered
colony morphology. It was theorized, based on the described
morphology of the ICM colony, that specific cells could be
separated and used for passaging and that these separated
cells might potentially result in the production of cultured
25   ICMs having the desired multilayer morphology.

      As noted, it was observed that the growing ICM cell
colony, while initially entirely multilayer, quite rapidly
flattens out to produce an epithelial sheet of cells having
two distinct populations of cells within the colony. The
30   first population is comprised on the outer perimeter of the
cell colony and possesses a multilayer structure and in-
cludes cells which possess the following morphological
characteristics:

          (i)   small cell size (cells ranging from about 10 - 20
35   μm in diameter, preferably less than 15 μm in diameter);
          (ii) observable cytoplasmic vesicles; and

(iii) small cytoplasmic/nuclear volume ratio (ranges from about 10/90 to about 50/50, preferably about 10/90 to about 30/70, and most preferably about 25/75.)

This outer section of the colony was also found to
5   stain strongly positive for AP activity. By contrast, the cells in the middle of the colony tend to be comprised of flattened epithelial-like cells which exhibit little or no AP activity.

Based on the observed AP activity and multilayer struc-
10 ture, the present inventors decided to selectively passage only or substantially only the cells in the outer perimeter of the cell colony and specifically the cells having small cytoplasmic volume/nuclear volume ratio, observable cyto-plasm vesicles and small cell size (defined *supra*) in the
15 hope that these cultured cells would produce additional multilayer CICM cell colonies having the desired morphology. However, this outcome was not at all assured. To the con-trary, it was possible that such passaging could have in-stead resulted in colonies consisting entirely of flattened
20 epithelial-like cells, particularly if the observed epithe-lial-like appearance and altered cell marker expression were a consequence of culturing ICMs *in vitro* over prolonged time or a consequence of ICM cell passaging.

Quite surprisingly, the present inventors discovered
25 that the selective passaging of cells comprised in the perimeter of the multilayer cell colony, possessing the above-enumerated morphological characteristics produced ICM multilayer colonies. Also, it was surprisingly found that these multilayer colonies contain cells which express cell
30 markers identically or substantially similarly to ICM of developing embryos. Moreover, it was found that the subject .method provides for the maintained production of CICMs which exhibit a morphology and express cell markers identically or substantially similarly to ICM of developing ungulate embry-
35 os, theoretically indefinitely. CICMs produced according to the invention maintain such properties for prolonged periods

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in culture, i.e., for at least one passage, after about 5 to
10 passages, and more preferably after about 10 to 50 pas-
sages.

     The selective passaging of cells having the desired

5   morphological characteristics may be accomplished by known
cell separation methods. For example, the multilayered
portion of the colony which constitutes the perimeter of the
colony may be separated from the middle portion of the
colony by physical means, e.g., using a glass pipette or a

10 needle. The large cell clumps which result can then be
further broken down by physical and/or chemical and/or
enzymatic means. For example, cell dissociation may be
effected by the use of enzymes, e.g., trypsin or pronase.
Alternatively, mechanical cell separation may be effected by

15 repeatedly pipetting large cell clusters or cell clumps
through a cell pipette or by use of a needle or razor blade
to cut large cell clumps into smaller groups. Preferably,
such chemical and/or mechanical cell separation will produce
CICM clusters suitable for passaging, i.e., which contain

20 about 5 to 100 cells.

     The separated cells used for passaging will preferably
consist of cells contained in the multilayer peripheral
section of the cell colony, preferably having the above-
recited morphological characteristics. However, in some

25 cases, even cells from the inner portion of the cell colony,
when treated according to the invention, are found to pro-
duce multilayer colonies after passaging onto a new feeder
layer.

     This is believed to occur because the cells revert back

30 to the desired multilayer colonies either because of the
passaging procedure or the reestablishment of cell-to-cell
.contact with the new feeder layer. However, the present
inventors do not wish to be bound by their belief.

     During passaging, it is essential that the small ICM

35 cells or cell clusters be placed into direct contact with
the feeder layer to prevent CICM colony differentiation. It

has been found that if the cells or cell clusters are grown without sufficient contact with feeder layers that this instead results in embryoid bodies. Such embryoid bodies may be observed in Figure 1 which is a photograph showing

5 cultured ICM cells grown without sufficient contact with a feeder layer.

To initiate direct contact, any method may be used which provides for direct cell contact between passaged CICM cells and feeder cell layer which is not degradative to the

10 CICMs and does not adversely affect colony production. The least efficient means is to simply allow the small clumps of cells to settle down on top of the feeder layer. It is more preferable to use methods which provide for more efficient cell-to-cell contact between the CICM cells and the feeder

15 layer. This has been found to result in a higher number of multilayer CICM cell colonies.

Any physical means which provides for enhanced physical contact between the CICM cells and the feeder cells, but which is not unduly degradative, i.e, does not adversely

20 affect the production of the desired multilayer type cells and CICM cell lines may be used. Methods for providing for increased direct contact of passaged CICMs and the feeder layer include, by way of example, the use of a pipette to press the individual clumps of cells onto the feeder layer;

25 the placement of CICM cell clusters under the feeder layer so that the cells are sandwiched between the feeder layer and the bottom of the dish; and the centrifugation of clumps of cells on top of the feeder layer, e.g., from between 100 and 5000 g for about 10 minutes to 5 hours to force the cell

30 clusters onto the feeder layer. These methods are merely exemplary of methods which may be used to force the CICM cell clusters into close contact with the feeder layer. Other methods may be substituted therefore provided that they do not adversely affect the formation of the desired

35 multilayer CICM cell colonies. As discussed above, it has also been found that passaging efficiency may further be

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-enhanced by passaging CICM cells together with some associ-
ated feeder cells onto the new feeder colony. Apparently
the presence of some of the feeder cells from the previous
passage enhances the percentage of CICM cell clumps which
5   result in new colonies.

In general, the above culturing procedure for culturing
CICM cells is applicable to any ungulate derived CICM cells.
For example, procedures initially found to be suitable for
culturing pig CICM cells have been found to be applicable to
10 bovine CICM cells. However, one observed difference is that
the majority of cells derived from bovine embryos are AP
negative whereas those derived from pig embryos are AP
positive. It is hypothesized that there may exist species
differences in AP expression in cows and pig ICMs. However,
15 this is unclear because the inventors were able to produce
one bovine cell line that was weakly AP positive and which
grows in small clumps. (*See* Figure 2).

Also, the bovine cells differ from the pig cells in
that the borders of the colony are not as well defined.
20 However, similar to the pig derived cells, the cells com-
prised in the perimeter of the colony are AP positive where-
as cells in the center of the colony tend to lose AP activi-
ty. This also may be appreciated upon review of Fig. 2.
Similar to pig derived CICMs, these cells are cytokeratin 18
25 negative.

As shown in the Examples, and described in more detail
infra, the subject CICMs are useful for the introduction of
heterologous DNAs. In particular, transgenic CICM cell
lines have been produced which contain within their genome a
30 heterologous DNA (beta-galactosidase DNA construct). Also,
recently the inventors obtained cells using the above-de-
scribed culturing methods which were somewhat different from
the described CICM cells in their morphology, ability to
differentiate, level of endogenous beta-galactosidase activ-
35 ity (higher), and ability to express beta-galactosidase DNA
constructs. Moreover, these cells were AP negative from the

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onset of culturing, whereas previous CICM cells of pigs
tended to lose AP activity over time during culturing.
Similar cells have been observed and propagated in CICMs
derived from cows. Although these cells lack AP activity,
5   they exhibit some characteristics in common with ICM cells.
Therefore, they may also be useful for production of trans-
genic cloned embryos, as well as the other described appli-
cations of CICMs.

       The present invention further provides another method
10   for providing cultured ICM cells and cell lines having the
desired morphological and cell marker characteristics, e.g.,
CICMs which are AP positive and cytokeratin 18 negative.
This second method will also involve obtaining and culturing
ICMs, preferably from blastocyst or preblastocyst stage
15   embryos of ungulates on feeder cell layer cultures. Prefer-
ably, the culturing techniques and passaging methods will be
effected as described above.

       However, the second method differs in the fact that the
cultured ICM cells will express, preferably only under
20   specific conditions, a differentiation inhibiting (DI) gene.
This is preferably accomplished by introducing into the ICM
cells, at some time during the culturing or passaging proce-
dures, a nucleic acid sequence which includes a differentia-
tion inhibiting gene, which is preferably expressed under
25   the control of an inducible or regulatable promoter.

       As noted above, DI genes refer to any gene or genes
which may inhibit cell differentiation in the CICM cell
colonies which do not adversely affect the isolation of CICM
cell lines having the desired morphological characteristics
30   and cell marker expression. The DI gene, preferably opera-
bly linked in proper reading frame to a regulatable or
inducible promoter may be introduced into the nucleus of
embryonic cells from which the CICM cell lines are derived
during passaging, or alternatively introduced into an estab-
35   lished CICM cell line.

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This is effected in a manner which results in the DI
gene, or transgene as it may be accurately described, being
integrated into the genome of the embryonic ICM or cultured
ICM cells or cell line. Methods for introducing desired
5   DNAs into mammalian cells and embryonic cells in particular
are known in the art and include by way of example microin-
jection, electroporation, lipofection, retroviral insertion,
Ca precipitation, and liposome insertion. To date, micro-
injection appears to be the most efficient means for intro-
10   ducing the DNA into CICM cell lines. However, it is expect-
ed that other methods will also be effective with appropri-
ate optimization.

Differentiation inhibiting genes suitable for use in
the invention include, e.g., tsA58 (See WO 91/13150), other
15   T antigens and oncogene products known to inhibit differen-
tiation (See WO 91/13150 for examples thereof), OCT3
(Okamoto et al., Cell, 60:461 (1990), Rosner et al., Nature,
345:686, (1990), LIF and the LIF receptor (Smith et. al.,
Nature, 336:688 (1988)). These DI genes are merely exem-
20   plary of those which may be used in the present invention.

The DI gene is preferably placed under the control of
an inducible or regulatable promoter. As noted, examples of
inducible promoters are well known in the art and include by
way of example the metallothionein promoter (metal ion
25   inducible), as well as the response elements for tetracy-
cline, interferon and steroid (See WO 94/29442; Kimura
et al., Cell, 44:261 (1986); Yarranton, Curr. Opm. Biotech,
3:506 (1992)).

After the transgene is integrated into the embryonic or
30   cultured ICM cells or cell line, and the resultant trans-
genic cells are established onto feeder cell cultures, the
DI gene or genes are turned on by inducing the particular
inducible promoter. This is typically effected by adjust-
ment of culturing conditions. For example, if the promoter
35   is the metallothionein promoter induction is effected by
introduction of a culture medium containing appropriate

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metal ions which induce ("turn on") the promoter. Thereby,
when the cells are cultured under induction conditions, the
cultured ICM cells should continually or for prolonged
periods in tissue culture maintain the desired CICM cell
5   morphology and gene expression characteristics. More spe-
cifically, the cells should exhibit the desired multilayer
cell colony morphology and express cell markers identically
or substantially similarly to ICMs of developing embryos,
i.e., the cells will generally be AP positive and cytokera-
10   tin (cytokeratin 18) negative. Thereby, problems such as
ICM cell colonies differentiating into flattened epithelial
sheets which are AP negative and cytokeratin 18 positive
(which are observed when ICMs are cultured under convention-
al conditions) should be minimized or even eliminated.
15   Moreover, this should also prevent the differentiation of
ICM cells during passage and during sustained culture peri-
ods.

The resultant cultured ICM cells and cell lines which
are obtained by the above-described methods possess numerous
20   usages. Most especially, these CICM cell lines may be used
to produce offspring which possess the CICM genetic makeup
in whole or part.

Chimeric offspring may be obtained by injecting the
CICM cells directly into the blastocoele cavity of recipient
25   embryos or combined with pre-blastocyst stage embryos. The
resultant chimeric embryos are then placed into a recipient
female. The resultant offspring should then have a CICM
genetic contribution to all organ systems including the germ
cells of the reproductive organ. Thereby, the chimeric
30   animal can pass the CICM genetics into subsequent genera-
tions of offspring. Also, the introduced CICM may have
.introduced in their genome a desired gene or genes. There-
by, chimeric offspring may be obtained which express a
desired gene or genes. For example, genes may be introduced
35   into CICMs which provide for enhanced livestock properties,
e.g., which encode hormones, which provide for disease

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resistance, (e.g., lymphokines, viral resistance genes, bacterial resistance genes), enhanced milk production, altered fat percentages, enhanced body weight, among other enhanced properties. Also, genes may be introduced which
5   encode for desired gene products, e.g., genes which encode products useful as human therapeutics or for xenotransplan- tation.

Also, the subject CICM cells may be used in nuclear transfer procedures to obtain nuclear transfer embryos,
10 fetuses and offspring. Nuclear transfer techniques are known in the literature and are described in many of the references discussed in the Background of the Invention. *See* in particular, Campbell et al., Theriogenology, 43:181 (1995); Collas et al., Mol. Reprod. Dev., 38:264-267 (1994);
15 Keefer et al., Biol. Reprod., 50:935-939 (1994); Sims et al., Proc. Natl. Acad. Sci. USA, 91:6143 (1994); Stice et al., Theriogenology, 41:301 (1994); Sims et al., Proc. Natl. Acad. Sci. USA, 90:6143-6147 (1993); WO 94/26884; WO 94/24274; and WO 90/03432 which are incorporated by
20 reference in their entirety herein. Again, if the CICM contributes to fetal germ cells, the CICM genetics can be passed onto subsequent generations of animals. Similarly, the CICM cells may be genetically engineered such that they have integrated into their genome a desired gene or genes,
25 e.g., genes which provide for enhanced livestock properties, or which encode for desired gene products, e.g., human therapeutics or other polypeptides.

Still further, differentiated cells, tissues or organs produced by nuclear transfer or obtained from chimeric
30 fetuses or offspring may be used in transplantation thera- pies. For example, nuclear transfer or chimeric fetuses derived from CICM cells containing an anti-rejection gene or genes may provide a source of hematopoietic cells useful to supplement or replace human hematopoietic stem cells. This
35 is potentially useful in immunocompromised patients, e.g., AIDS patients or other diseases affecting hematopoietic stem

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cells. Also, stem cells may be useful in treatment of
Huntington's disease, Parkinson's disease and Alzheimer's
disease. Moreover, pancreatic cells may be useful in
diabetes treatments. Further transplanted liver cells may
5   be useful for the treatment of liver diseases. Alternative-
ly, whole soft organs may potentially be transplanted from
genetically altered CICM-derived ungulates into humans (See
Durling et al., Curr. Omp. Immunol., 6:765 (1994)) incorpo-
rated by reference in its entirety herein.
10   Also, the subject CICM cell lines may be used as an
in vitro model of differentiation, in particular for the
study of genes which are involved in the regulation of early
development.
         This is only exemplary of potential applications of
15   CICM cell lines obtained according to the present invention.
         The invention will now be described in more detail in
the following Examples.

                            EXAMPLE 1
20   Production of CICM cell lines from pre-blastocyst and
blastocyst stage pig embryos were conducted using the fol-
lowing general protocol. First, primary cultures of embry-
onic fibroblasts were obtained from 12-16 day old murine
fetuses. After the head, liver, heart and alimentary tract
25   were aseptically removed, the embryos were minced and incu-
bated for 30 minutes at 37°C in prewarmed trypsin EDTA
solution (p.05% trypsin/0.02% EDTA; GIBCO, Grand Island,
NY). Fibroblast cells were plated in tissue culture dishes
and cultured in alpha-MEM medium (BioWhittaker, Walkers-
30   ville, MD) supplemented with 10% fetal calf serum (FCS)
(Hyclone, Logen, UT) penicillin (100 IU/ml) and streptomycin
(50 μg/ml). Three to four days after passage, embryonic
fibroblasts, in 35 x 10 Nunc culture dishes (Baxter Scien-
tific, McGaw Park, IL), were treated with mitomycin C
35   (10μg/ml; Sigma) in supplemented alpha MEM for a minimum of
three hrs. The fibroblasts were grown and maintained in a

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humidified atmosphere with 5% CO_2 in air at 37°C. Only
culture plates which had a thick confluent monolayer of
cells were used to culture the CICM cell lines. The charac-
teristics of the feeder layer is an important factor in
5   obtaining and propagating undifferentiated CICM cell lines.
         Porcine embryos were surgically collected from the
reproductive tract post mortem or during surgical laparoto-
my. The ICM of blastocyst stage embryos were either par-
tially separated from the trophoblast cells using a cutting
10   needle, incubated in trypsin or pronase, or left intact.
The ICM and at least a portion of the trophectoderm was
plated directly onto the mitomycin C blocked fibroblast
cells often using a glass pipette to initiate contact
between the ICM and the fibroblast feeder layer. The CICM
15   cell lines were maintained in a growth medium consisting of
alpha MEM supplemented with 10% FCS and 0.1 mM beta-mercap-
toethanol (Sigma). Growth medium was exchanged every two to
three days. Initial colonies were observed by the fourth
day of culture and could be passaged any time after the
20   fifth day. Only cells having the following three morpholog-
ical features were isolated for passage: a small cytoplas-
mic/nuclear volume ratio, cytoplasmic vesicles, and gener-
ally small individual cells (less than 15 μm in diameter).
These cells are often isolated from multilayer portions of
25   the colony which maintained direct contact with the feeder
layer. The portions of the colony that met this criteria
were often AP positive and cytokeratin negative upon passage
onto a new feeder layer.
         When the ICM or the passaged CICM cells initially
30   attach to the feeder layer, multiple layer colonies consist-
ing of small cells are visible after two days in culture.
This can be seen in Fig. 3. The multilayer colonies are AP
positive and cytokeratin negative (See Figs. 4 and 5).
However, some of the colonies begin to form an epithelial-
35   like sheet of cells. The epithelial-like cells are AP
negative and cytokeratin positive (Figs. 6 and 7). In

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addition, the multilayer colonies often begin to flatten into epithelial sheets of cells as they propagate *in vitro* (Fig. 8).

5   It was observed by the inventors that a growing multi-layer colony begins to flatten out, forming an epithelial sheet of cells having two distinct populations of cells within the colony. The first population resides in an area around the perimeter of the colony. This section of colony is multilayered and the individual cells are small and

10   possess cytoplasmic vesicles (Fig. 8). This area also stains positive for AP activity. The other area of the colony contains the flattened epithelial-like population of cells. These cells tend to be in the middle of the colony. In this population of cells, individual cells and cell

15   borders can be observed when viewing the colony under a microscope (Fig. 8). Again, these cells have very little AP activity or none at all.

       It was theorized that to maintain the desired multi-layer type cells, preferably, only the cells around the

20   perimeter would be selectively passaged to produce addition-al multilayer colonies.

       This is probably accomplished by using a glass pipette razor or a needle to cut out the multilayer portions of the colony (perimeter cells). The large groups of cells can be

25   broken down further either by mechanical separation or by using trypsin (0.05% trypsin/0.2% EDTA) along with the mechanical separation. Mechanical separation is conducted by repeatedly pipetting large clumps of cells up and down through a small bore pipette. Alternatively, a needle or

30   razor blade can be used to cut the large group of cells into smaller groups. It was surprisingly found that CICM clumps obtained by all these methods (5 to 100 cell) can then be passaged onto new feeder layers to produce cultures having the desired multilayer morphology. In some occasions even

35   cells from the inner portion of the colony when treated in this same manner, produced multilayer colonies after passage

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onto new feeder layers. It is hypothesized that these cells reverted back to the multilayer colonies as a result of either the passage procedure or the reestablishment of cell to cell contact with the new feeder layer.

5   It was observed that the small clumps of cells must be placed back in direct contact with the feeder layer to prevent CICM colony differentiation. By contrast, cells grown without contact with feeder layers form embryoid bodies (Fig. 1). There are several methods used to re-

10 initiate feeder layer contact. The least efficient means is to allow the small clumps of cells to settle down on top of the feeder layer after the cell culture plates are placed back in the incubator. A preferred method involves forcing cell to cell contact between the CICM cells and the feeder

15 layer. This results in a higher number of newly established CICM cell colonies. One way to force cell to cell contact is to use a pipette to press the individual clumps of cells down on top of the feeder layer. Another method is to place the clump of CICM cells under the feeder layer so that the

20 cells are forced between the feeder layer and the bottom of the culture dish. Still alternatively, clumps of cells on top of the feeder layer can be centrifuged between (100 and 5000 g) for (10 min to 5 hrs) to force the cells down on top of the feeder layer. Essentially, any method which forces

25 the passaged clumps of CICM cells into close contact with the feeder layer results in the production of CICM cell colonies having the desired multilayer morphology. Also, as discussed previously, passaging efficiency may be further enhanced by passaging CICM cells along with some associated

30 feeder cells onto the new feeder layer.

                              EXAMPLE 2

        CICM cells obtained according to Example 1 were used for insertion of heterologous DNA's. Specifically, these

35 cells were microinjected with linear as well as supercoiled DNA constructs containing different promoters placed in

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front of either the beta-galactosidase gene and/or the
neomycin phosphotransferase gene. The specific promoters
used were the cytomegalovirus promoter (CMV promoter),
phosphoglycerate Kinase promoter (PGK promoter), mammary
5   promoter (MAM promoter), reCMV promoter and chicken beta
actin promoter. These gene constructs were diluted in a
buffered solution (containing 80mM KCl and 70mM HEPES.)
However, other buffers may readily be substituted therefore,
such as Tris EDTA. The concentration of the DNA constructs
10 in solution ranged from 5 to 10μ g/ml. However, concentra-
tions ranging from 0.1 to 100 μg/ml should be effective.
These DNA preparations were then microinjected into cultured
CICM cells obtained according to Example 1.

    In this procedure, the individual CICM cells within the
15 colonies were located by viewing the colonies through an
inverted microscope. Thereafter, the cell membrane alone or
additionally the nuclear membrane was punctured using a
small injection needle containing the DNA preparation. The
opening in the needle was about 1 μm. Such a diameter
20 needle was selected to help prevent CICM cell lysis. More-
over, the microinjection procedure was facilitated by the
use of a micromanipulator which was attached to the inverted
microscope.

    After the injection pipette was introduced into the
25 nucleus, approximately 700 copies of the DNA were released
into the nucleus. The micropipette was then removed from
the cell. This process was repeated for other cells in the
CICM colony. Ideally, 1000 cells may be microinjected per
hour.

30   The results obtained using the different promoters are
summarized in the following table.

    Heterologous Gene Expression in Microinjected CICM Cells

promoter	expression two hours after injection	expression five days after injection
CMV	+++	++

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PGK	++	none
reCMV	++	not tested
MAM	++	not tested
C-Actin	+	not tested

5

The above results demonstrate that vectors containing
each of the tested promoters result in cells which express
the inserted heterologous DNA. It was also observed that
10 microinjection with several constructs did not provide for
any additive effects on gene expression.

Figure 9 is a photograph of microinjected pig CICM
cells containing a CMV beta-galactosidase construct detected
by X-gal staining. Nests of cells expressing b-galactosi-
15 dase may be seen. This indicates that the β-galactosidase
gene has been effectively incorporated into the cell genome
and is being transmitted and expressed in daughter cells.

The above results were obtaining using pig CICM cells.
Moreover, using similar methods, bovine CICM cells were also
20 injected with CMV and PGK-beta-galactosidase constructs.
Both DNA constructs resulted in recovery of cells which
express beta-galactosidase.

Thus, these results demonstrate that CICM's cultured
according to the invention may successfully be used for
25 integration and expression of desired heterologous DNA's.
Also, these gene expression characteristics may be passed
onto daughter cells.

EXAMPLE 3

30   In this culturing method, a DI gene is conditionally
expressed in CICM cell colonies to prevent cell differentia-
.tion. The cell passage and culture techniques are the same
as in Example 1 with the difference being the genetic makeup
of the CICM cells. Specifically, a DI gene is introduced
35 into the nucleus of embryonic cells from which the CICM cell
lines are derived or into an established CICM cell line.

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The transgene is then integrated into the genome of the CICM
cells. Any known methods of introducing transgenes into
embryonic cells can be used, including by way of example
microinjection, electroporation, retroviral insertion, Ca
5   precipitation, and liposome insertion.

The inserted transgene is expressed under the control
of an inducible promoter. Inducible promoters include,
e.g., response elements such as tetracycline (WO 94/29442),
interferon (Kimura et al., 1986), steroid and metallothio-
10 nein (reviewed by Yarranton, 1992). Accordingly, the DI
gene is inserted such that it is operably linked, in proper
reading frame, with an inducible promoter.

After the chimeric gene construct is integrated into
the genome of the embryonic cells and the multilayer ICM
15 cell colony is established, the DI gene is expressed by
inducing the inducible promoter. This expression provides
for the ICM cell colony to continuously, or for prolonged
time in tissue culture, maintain the desired multilayer
morphology and to express genes consistent with ICM of
20 developing embryos. Thus, problems such as cells differ-
entiating into flattened epithelial sheets that lose their
AP expression and express cytokeratin 18 are minimized or
even avoided altogether. This method is also useful in
preventing differentiation of cells during long term culture
25 periods.

While the invention has been described with respect to
certain specific embodiments, it will be appreciated that
many modifications and changes thereof may be made by those
skilled in the art without departing from the spirit of the
30 invention. It is intended, therefore, by the appended
claims to cover all modifications and changes that fall
within the true spirit and scope of the invention.

WHAT IS CLAIMED IS:

1.   A method for producing a cultured inner cell mass
(CICM) cell which comprises the following steps:
5   (i)   obtaining an ICM of a blastocyst or ICM progeni-
tor cells from preblastocyst stage embryo;
         (ii) culturing said ICMs or ICM progenitor cells on a
feeder layer culture under conditions which provide for the
formation of multilayer cell colonies;
10   (iii) identifying from among the cells contained in the
cultured ICM cell colony those cells which exhibit the
following properties:
              (a)   small cytoplasmic/nuclear volume ratio;
              (b)   cytoplasmic vesicles;
15   (c)   small individual cells relative to rest of
the cell colony;
         (iv) separating one or a cluster of said identified
cells from the rest of the cell colony; and
         (v)   passaging said separated ICM cells or ICM progen-
20 itor cells onto another feeder layer culture under condi-
tions whereby there is at least some physical contact be-
tween the feeder cell layer and the separated cells or cell
cluster.

25 2.   The method of claim 1 wherein said ICM obtained in
step (i) include at least a portion of the trophectoderm.

3.   The method of claim 1 when the feeder cell layer com-
prises fibroblasts.
30
4.   The method of claim 3 wherein said fibroblasts are
murine embryonic fibroblasts.

5.   The method of claim 4 wherein said murine embryonic
35 fibroblasts are primary cells.

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6.   The method of claim 3 wherein said fibroblasts comprise a thick confluent monolayer.

7.   The method of claim 1 wherein the method for producing for physical contact of the separated cultured ICM cells and the feeder cell layer is selected from the group consisting of (i) placing the separated ICM cells on top of the feeder cell layer, (ii) pressing CICM cell clusters onto the feeder layer, (iii) placing CICM cell clusters between the feeder cell layer and the culture dish, and (iv) centrifuging the CICM cells or cell cluster onto the feeder cell layer.

8.   The method of claim 1 wherein the separated identified cells are comprised in the outer portion of the multilayer CICM cell colony.

9.   The method of claim 8 wherein the separated cells further include some cells comprised in the inner epithelial-like portion of the cell colony.

10.   The method of claim 1 wherein the identified cells are separated from the colony by at least one of the following: physical or chemical or enzymatic means.

11.   The method of claim 10 wherein said physical means comprises the use of a glass pipette, hypodermic needle or razorblade.

12.   The method of claim 11 which further comprises treatment of separated cells with trypsin or pronase.

13.   The method of claim 1 wherein the separated cells comprise a cluster of about 5 to 100 cells.

14.   The method of claim 1 wherein steps (ii) through (v) are repeated.

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15. The method of claim 1 wherein the cytoplasmic/nuclear ratio ranges from about 10/90 to about 50/50.

16. The method of claim 15 wherein said ratio is about 25
5   to 75.

17. The method of claim 1 wherein the cells range in size from about 10 to 20 microns in diameter.

10 18. The method of claim 2 wherein the cells are less than about 15 microns in diameter.

19. Cultured inner cell mass cells (CICM cells) derived from the ICM of a blastocyst or derived from ICM progenitor
15 cells obtained from preblastocyst ungulate embryo wherein said cultured inner cell mass maintain in culture the mor-phological characteristics and express cell markers identi-cally or substantially similarly to ICMs of developing ungulate embryos.
20

20. The CICMs of claim 19 which express alkaline phospha-tase.

21. The CICMs of claim 19 which do not express cytokeratin
25 18.

22. The CICMs of claim 19 wherein said cells exhibit a multilayer colony morphology in tissue culture.

30 23. The CICMs of claim 21 wherein said multilayer colony morphology comprises an inner epithelial-type portion and a multilayer portion which substantially surrounds said epithelial-like portion.